

AN ANTIBIOTIC FROM *BACTERIUM COLI*.

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A CONSIDERABLE literature has collected during the past 50 years on the antagonistic effects produced by *Bact. coli*. As a full review of these contributions will be published elsewhere, it is proposed to mention now only those considered the most important. In 1904 Eijkmann performed experiments which led him to conclude that several coliform bacteria produced thermolabile, inhibitory substances which were diffusible, but would not pass through a porcelain filter. He could not detect these inhibitors in broth cultures. Conradi and Kurpjuweit (1905) also claimed to demonstrate a similar heat-labile substance, though their remarkable statement that cultures could be diluted 3200 times and still stop bacterial growth seems hardly credible. Other authors such as Moro and Murath (1906), Leuchs (1907) and Chatterjee (1909) considered that *Bact. coli* elaborated a thermolabile antibiotic. Nissle (1916) published work on the "antagonistic index" of *Bact. coli* versus *Salm. typhi*. He thought that "active" strains produced something which inhibited the growth of a number of disease-producing bacteria. Active strains were sold under the name of "Mutaflor" for the treatment of various intestinal infections. McLeod and Govenlock (1921) found that broth filtrates obtained after the growth of *Bact. coli* would not support growth of *Bact. coli* nor certain other organisms, but if the culture media were diluted 1 part in 5 of water they again became capable of sustaining growth, thus the inhibition was not due to exhaustion of the medium. McLeod and Gordon (1922) showed fairly conclusively that a similar inhibitory effect produced by pneumococci was due to hydrogen peroxide formation. They found that some other organisms also formed hydrogen peroxide; though the strain of *Bact. coli* they examined in this respect did not do so, it seems quite possible that the strain studied by McLeod and Govenlock (1921) did produce hydrogen peroxide. Van der Reis (1921, 1922) described the destruction of *C. diphtheriae* by *Bact. coli* a thermolabile, non-dialysable and non-filtrable material being the active agent. Pesch and Zschocke (1922) also found that *Bact. coli* killed *C. diphtheriae*. Gundel and Habs (1927) and Gundel (1930) believed that *Bact. coli* produced thermolabile antibacterial substances, and Gundel (1930) extracted a "lipoid" from *Bact. coli* which was active against staphylococci and *Bact. coli*.

There are many other papers describing inhibitions produced by *Bact. coli*, but those of Gratia (1925 and 1932) are, from the point of view of the present paper, the most important. He noted that one strain of *Bact. coli* (called by him Coli V), which was very virulent for rabbits and guinea-pigs, produced something in the medium on which it grew which showed inhibitory action towards another *Bact. coli* strain (Coli ϕ) even when the medium was diluted as much as 1000 times. He deduced that as the active substance was freely diffusible through cellophane and was very stable, it was not a bacteriophage. It retained its

activity for months in sealed tubes, and withstood boiling for 1 hour and heating to 120° C. for half an hour. Later it was shown to be precipitated by acetone. The Coli V apparently lost for a time its capacity to produce the substance, but eventually recovered it spontaneously. A full account of his experiments was furnished by Gratia in 1932, when he illustrated the inhibition. He noted that although most of the Coli ϕ were inhibited around the colony of Coli V on an agar plate some resistant organisms grew up in the inhibited zone, and that though the development of Coli ϕ in broth might be stopped for some hours full growth eventually appeared. Thus there seemed to be bacteria of the Coli ϕ strain which were resistant to the Coli V substance. Gratia showed that the faster the Coli V grew the better the yield of the substance, and that if it was left in contact with the Coli V bacteria for 5 days it was destroyed. It had slight activity against *Sh. shigae*; whether any other organisms were tested is not stated.

Guelin (1943) described a substance (called L 36) very similar to that of Gratia. A special strain of *Bact. coli* was found to produce in the broth in which the organism had grown a substance which was thermostable and could be filtered. This substance was found to lyse very young cultures of *B. paradyenteriae* and another strain of *Bact. coli*, but resistant forms seemed to be readily produced. The material was not a bacteriophage. Calcium ions were apparently necessary for the development of the substance, but not for its lytic action.

Demelenne-Jaminon (1940) reported being able to separate two forms from the resistant strains of Coli ϕ of Gratia. One form grew in large colonies, 2-3 mm. in diameter, while the other formed minute colonies seen only with difficulty after 24 hours' incubation. On subculture both bred true for more than a year. Both had the characteristics of a true *Bact. coli*. The proportion of resistant bacteria in Coli ϕ was 0.004 to 0.005 per cent, and among these the large colony type was about twice as numerous as the small.

In 1945 Wiedling described the existence of a thermostable antifungal substance produced in fluid medium by a strain of *Bact. coli*. He was led to notice this by the fact that *Penicillium notatum* grew poorly on a medium which had been accidentally contaminated by *Bact. coli* (though there was no appreciable reduction of the nutrients). The inhibitory substance was not destroyed by autoclaving at 120° C. for 20 minutes. It was not isolated, nor were its properties further discussed.

A thermolabile lytic substance was observed by Zamenhof (1945) to be formed by a smooth strain of *Bact. coli*. This lysed specifically the cells of another rough strain, but was quite inactive against several other strains of *Bact. coli* as well as staphylococci and Gram-positive bacilli. The lytic substance was not a phage nor a specific polysaccharide. It was destroyed by heating for 15 minutes at 81° C., but was practically unaffected at 61° C. for 1 hour. It was stable for 1 month at 25° C. It was not bacteriostatic but bacteriolytic. The author stressed the high specificity of the action of this substance.

The immediate starting-point of the present work was the observation of Goldsworthy and Florey (1930) that a strain of *Bact. coli* obtained from cat faeces produced something which diffused through agar and inhibited the growth of *M. lysodeikticus*.

After this paper had been prepared for publication, it was learned (private communication to H. W. F.) that observations on highly specific inhibitions among

coliform and other bacteria have been made recently by Professor A. Gratia, Dr. P. Fredericq, and their colleagues. Several communications on the subject have been made to learned societies and though in process of publication, have not yet appeared. It is understood that the production of several different inhibitors (named "colicines") by strains of *Bact. coli* has been proved, and that a system of classification is being worked out. Professor Gratia has kindly examined the *Bact. coli* strain CF 1 used in this work, and reports that it appears to be identical in all respects with his Coli V. To avoid later confusion, we are adopting his generic name "colicine" for our substance, but have otherwise not altered the text of this paper.

EXPERIMENTAL.

Although an examination of 200 strains of *Bact. coli* of human origin selected at random showed that seven of these produced inhibitors (Jennings, unpublished), the strain studied in the present paper was not isolated from human material, but, like that of Goldsworthy and Florey (1930), from cat faeces.

From the first specimen of faeces examined, one coliform organism was selected which on glucose agar gave good and quantitatively similar inhibition against *Staph. aureus*, *Bact. coli*, *B. subtilis*, *Ps. pyocyanea*, and *C. xerosis* (Fig. 1). On Lemco or heart agar, only *Bact. coli* was affected (Fig. 2). Further experiments strongly suggested that in the presence of glucose, hydrogen peroxide was formed, but that under certain other conditions, a more specific inhibitor appeared which was responsible for the selective inhibition of *Bact. coli*. The organism was called strain "CF1," and the specific inhibitor it forms is, as explained above, in this paper being called "colicine." The organism was identified* as an "atypical Type I *Bact. coli*," with the fermentation reactions shown in Table I.

TABLE I.—*Fermentation Reactions of Bact. coli Strain CF1.*

Lactose	.	Acid, gas	Indol	.	Positive
Glucose	.	"	Litmus milk	.	Acid
Maltose	.	"	Gelatin	.	Not liquefied
Mannitol	.	"	Voges-Proskauer	.	Negative
Sucrose	.	Negative	Methyl red	.	Positive
Salicin	.	"	Citrate utilization	.	Negative
Dulcitol	.	Acid, gas	MacConkey 37°	.	Acid, gas
Sorbitol	.	"	" 44°	.	—

Preliminary experiments.

Although a good inhibition was invariably obtained by the streaking technique on heart extract agar and Lemco agar with or without various additions, several attempts to obtain the inhibitor in liquid culture, with various media and under

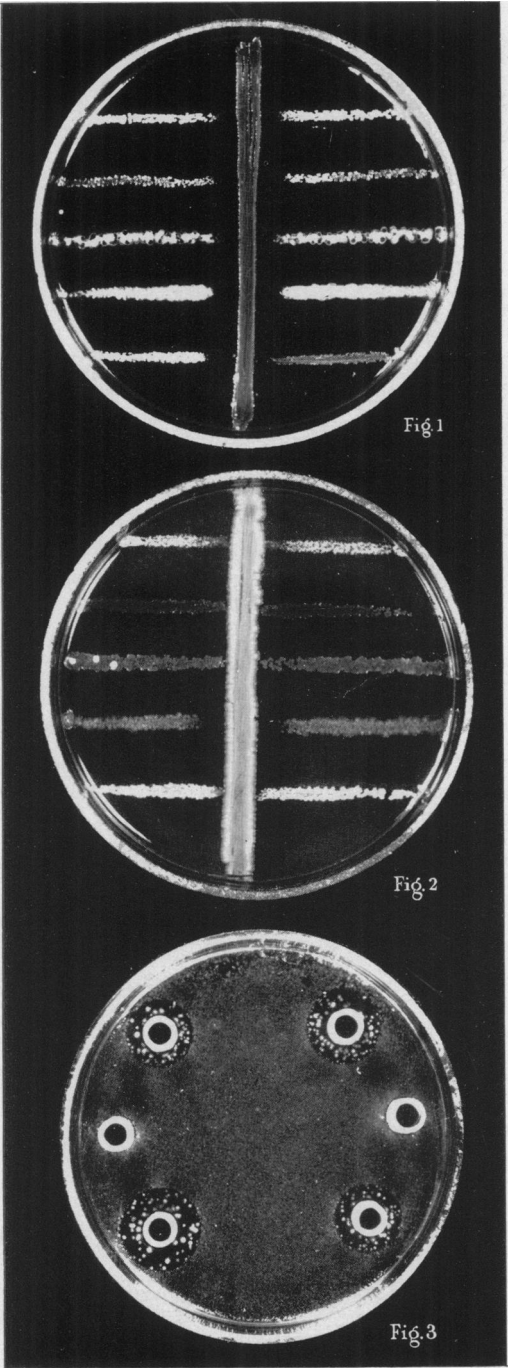
* We are indebted for identification to Dr. Joan Taylor of the Emergency Public Health Laboratory Service, Oxford.

DESCRIPTION OF PLATE.

FIG. 1.—Streak of *Bact. coli* CF1 on glucose agar. Cross-streaked with 1 in 1000 dilutions of broth cultures of *Staph. aureus*, *Bact. coli*, *B. subtilis*, and *Ps. pyocyanea*, and with a 1 in 10 dilution of *C. xerosis*.

FIG. 2.—Streak of *Bact. coli* CF1 on heart agar. Cross-streaks same as in fig. 1.

FIG. 3.—For explanation see text.



different conditions, were almost fruitless; at best only a trace of inhibition was obtained when the culture fluid was tested on a cylinder-plate. On the assumption that the formation of the inhibitor might require plentiful oxygen, the medium was used in thin layers or was shaken continuously, but little or no activity was produced. These liquid culture experiments were complicated by the fact that all viable organisms had to be removed from the liquid before testing, otherwise it was found that an inhibition might result from their growth during the test either in the fluid or on the agar inside the cup. In these unsuccessful tests this necessary sterility was achieved by centrifuging the suspensions, and then filtering the supernatant through a Gradocol membrane or a fritted glass filter. Seitz filtration and heating were not used because of the possibility that the substance, whose properties were not known, might be adsorbed on the filter or be destroyed by heat. (Later, Seitz filtration was found to hold back almost all the activity of this substance.)

Selection of test organism.

In the streaking tests, the ordinary laboratory strain of *Bact. coli* appeared to be completely inhibited. When this same organism was used for seeding plates for cylinder-plate assays there appeared in the good zones of inhibition which were obtained with broth from cellophane sacs (see below), a few discrete resistant colonies (Fig. 3), similar in appearance to those described by Gratia (1925, 1932) and Demelenne-Jaminon (1940). The reason for this appeared to be that the original culture was not homogeneous in respect of its sensitivity to colicine, for plates seeded with a culture derived from one of the resistant colonies showed no inhibition at all, whereas plates seeded with cultures from other single colonies isolated from the original test *Bact. coli* gave zones of inhibition completely clear of resistant colonies (Gratia, 1932). One of these freshly isolated sensitive strains was used for all subsequent tests and assays, and over a period of several months no resistant colonies appeared.

Method of assay.

The cylinder-plate method (Heatley, 1944) was used, the plates being surface-seeded with a 1 in 1,000 dilution of the sensitive *Bact. coli* described below. The assay value was little affected by the density of seeding. Though sharp zones of inhibition were obtained, the curve relating zone diameter to concentration of colicine was rather flat, so that the accuracy to be expected was correspondingly less.

Production of Colicine.

Cellophane sac experiments.

Gratia (1925) fashioned a sheet of cellophane into the form of a bag, which was filled with broth and immersed in a crystallizing dish also containing broth. When the broth outside the sac was inoculated with his "V" strain of *Bact. coli* the active substance formed passed freely into the broth inside, which remained sterile. With this experiment in mind it was shown that strain CF1 would grow well on the surface of a sheet of sterile cellophane laid on the surface of an agar plate. The inhibitor passed through the cellophane and after the cellophane and the bacteria on its surface had been stripped off there was good inhibition of a sensitive organism planted on the surface of the agar. This technique has

also been described by Gratia (1944), though his paper did not come to hand till after the experiments described below had been completed.

Based on this discovery another attempt was made to obtain the inhibitor in a fluid medium, in order to facilitate extraction.

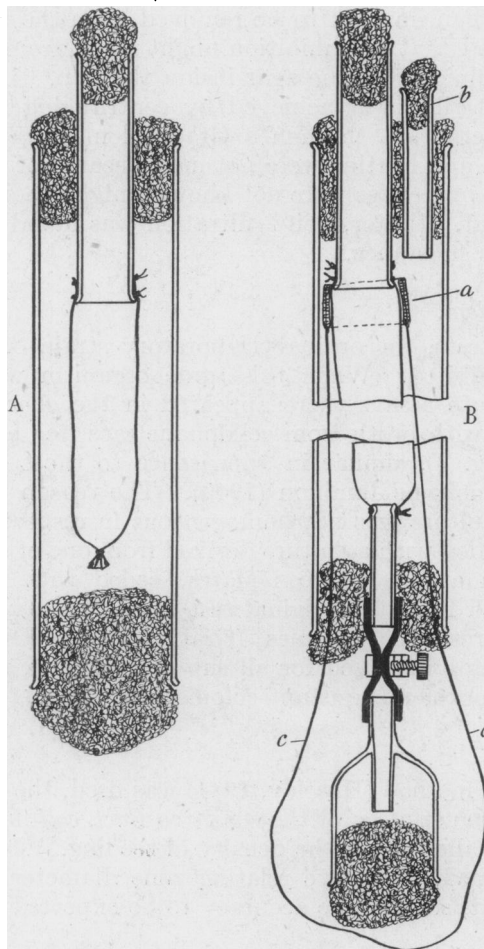


FIG. 4.—For explanation see text.

Cellophane sausage-casing sacs, tied onto glass tubes plugged with cotton-wool, were filled with Lemco broth and suspended in wide glass tubes plugged with cotton-wool at top and bottom (Fig. 4A). The whole assembly was sterilised by autoclaving, and when cool, the outside of the sac was inoculated with CF1 by stroking with a sterile swab dipped into a broth culture of the organism, the swab being introduced at the bottom of the tube after removal of the cotton plug.

After 24 hours' incubation at 37° C., the sterile broth inside the sac invariably gave a well-defined zone of inhibition of 16–18 mm. diameter on a cylinder-plate.

The medium could be replaced at this stage by fresh medium, and after 24 hours' further incubation (and without any further seeding or interference with the bacteria on the outside of the sac) another crop of active broth could be harvested. This replacement could be repeated many times with no apparent alteration in the yield of inhibitor, and it afforded an easy way of testing different media by putting in the sac, first broth, then a test medium, then broth again, then another test medium, and so on. Several modifications of the cellophane sac set-up were devised.

E.g., a tube tied into the bottom of the sac for withdrawing medium is shown in Fig. 4B, which also shows the arrangement used for sowing long sacs. After sterilising, the cloth cuff *a* is moistened with a few drops of broth culture (introduced through the small tube *b*), then slowly pulled down the length of the sac by means of two threads *c* passing through the plug at the bottom of the tube. After pulling out, the threads are cut off close to the plug and touched with a drop of lysol. An arrangement by which medium was continually allowed to drip into the top of the sac and run off from the bottom at the same rate through an automatic syphon was not found to have any advantages over the static arrangement, and was certainly more trouble.

Attempts to increase the scale, e.g. by suspending many sacs in a large bin, or laying them flat in trays, and seeding by means of a spray, were not very satisfactory, but it is worth mentioning that sacs filled with broth and tied off at both ends could be autoclaved without bursting and without much loss of broth.

A more practical method of increasing the scale was to cover a thin layer of broth in an enamel tray with cellophane, pleated so that the whole surface of the broth was covered, and extending well over the edges of the tray. The upper surface of the cellophane, which was sown with bacteria by a spray, was protected from contamination by a single thickness of lint secured by a draw-string. Stretched zigzag wires clipped over the edges of the trays supported the lint and prevented it from sagging unduly. The unwetted parts of the cellophane became brittle after autoclaving, but tearing during harvesting and replacement of the medium were avoided by directing a jet of steam onto the area of the cellophane which was to be unfolded.

Production of inhibitor on media other than Lemco.

Various adjuvants to the plain Lemco broth were tried in cellophane sac experiments in the hope of increasing the yield, but the addition of Marmite, corn steep liquor, gelatine digest, casein digest, lactose, lactate, extra peptone, extra Lemco, or extra glucose, made no appreciable difference. Dilution of the broth with water lowered the yield. Heart extract broth gave about the same yield as Lemco broth. Various synthetic media were tried. When the plate streaking test was carried out on a synthetic agar containing only glucose, KH_2PO_4 , MgSO_4 and ammonium sulphate or asparagine, good inhibition against several different bacteria was obtained (presumably due to hydrogen peroxide), but the same medium in sacs gave no inhibition at all, even against the sensitive *Bact. coli*. It was noted that in Lemco and heart extract broth, in which activity

was regularly obtained, the pH was always above 8.0, whereas that of the synthetic media was at or below 6.5. Addition of alkali, buffer, calcium carbonate or magnesium trisilicate to various media, during or at the beginning of an experiment, and with continuous agitation in the case of the solid reagents, gave no activity. When lactate was substituted for glucose with the object of raising the pH during fermentation, a definite, though small yield was obtained on three occasions, but after this could not be repeated, though several tests were made and a number of different samples of lactic acid were tried.

Aeration experiments.

It was puzzling that a good yield of inhibitor should be obtained when the bacteria were separated from the medium, whilst little or none was obtained when the bacteria were actually grown in it—especially so when it was found that prolonged incubation of a heavy suspension of living CF1 bacteria with a concentrate of colicine, followed by heat killing of the cells, did not diminish the activity at all. The experiences with cellophane again brought up the idea that proper aeration might be the key to the problem, although the results of early experiments with shaken cultures in thin layers of medium did not support it. The idea was fruitful, for when much more intensive aeration was produced by blowing a vigorous stream of air through inoculated Lemco broth, a good yield of inhibitor was formed—approximately the same as that obtained in cellophane sacs. Samples of these aerated broths were before assay placed in a closed phial, and completely immersed in a boiling water bath for about 5 minutes to kill the bacteria. It was shown that even much longer heating did not diminish the antibacterial activity.

Routine production.

The final procedure adopted for the production of colicine was the following :

Into rectangular porcelain vessels of the type used in this laboratory for the production of penicillin (Abraham, Chain, Fletcher, Florey, Gardner, Heatley and Jennings, 1941), were placed two litres of nutrient broth of the following composition :

Lab. Lemco	10 g.
Bacteriological peptone (Eupeptone or from Parke, Davis & Co.)	10 g.
Tap water	1 litre

The pH was adjusted to 7.0–7.2, and the liquid steamed or boiled for 30 minutes, then filtered. The pH was then adjusted to 6.8.

1–2 ml. of lard oil or arachis oil were added to each vessel to prevent foaming. The vessels, which were kept upright throughout, were closed by a cotton-wool plug through which a glass tube passed to the bottom of the vessel; the outer end of the tube was wrapped in lint or paper. The vessels were autoclaved for 30 minutes at 1 atmosphere pressure, then transferred to a water bath at 37° C. They were connected, with aseptic precautions,

to a separately sterilised train comprising (a) an air steriliser (a glass tube 3 cm. in diameter, by 40–50 cm. long, tightly packed with non-absorbent cotton-wool) connected to (b) a manifold, from the side tubes of which connection was made with (c) wash bottles containing water and a little oil. The outlet tubes of these wash bottles were wrapped in lint during the sterilising, and were subsequently connected, with aseptic precautions, to the vessels. The wash bottles also were immersed in the water bath and served two purposes, namely, moistening the air blown through the vessels, which prevented much evaporation from the latter, and providing a guide to the rate of aeration.

The vessels were seeded by tipping into each a small 24-hour Lemco broth culture of CF1, and aeration was begun, the stream of air being as fast as the wash bottles would allow. After about 16 hours the vessels were disconnected and steamed in the autoclave for 10–15 minutes to kill the bacteria, which were removed by passing the broth through a Sharples centrifuge.

Extraction.

From the clear broth, of which the pH was usually about 8.5, the inhibitor was removed by shaking with two successive portions of 0.5 per cent and 0.3 per cent respectively of "Farnell Grade 14" charcoal, which were separated on Buchner funnels, with the help of Celite. One-half to one-quarter of the amount of "Darco G 60" or "Puractim M" charcoal was as efficient as the amount and grade of charcoal actually used, but at that time these more effective grades were not obtainable in bulk.

The charcoal residues were briefly washed with water whilst on the funnels, then were boiled for a few minutes with 80 per cent alcohol, which removed a certain amount of yellowish inactive substance. This alcohol washing was usually repeated, then the charcoal was spread out to dry. The inhibitor was eluted from it with glacial acetic acid, which, apart from phenol and possibly pyridine, was the only effective eluent of the many which were tried. The charcoal and glacial acetic acid were mixed to a thick cream, then poured on to a Buchner funnel to which suction was applied for about half an hour, whilst at intervals the cake was firmly pressed down to obliterate cracks. Suction was stopped and more acetic acid was poured on to the cake, which slowly imbibed it. After a few minutes suction was again applied for half an hour, then the whole cycle was repeated three or four times, or until the filtrate was only faintly coloured. Usually the second, presumably weaker, batch of charcoal was eluted first, and the filtrate from this used for the elution of the first batch. The eluates were concentrated to a syrup by vacuum distillation, preferably in separate batches so that prolonged heating of any of the material was avoided. The syrup was ground up with sand and a large volume of absolute alcohol was gradually added. The resulting flocculent precipitate did not settle as readily as the sand, and could be poured off, with alcohol washings, into centrifuge tubes, and spun down. The alcoholic supernatant, carrying most of the acetic acid, was poured off, and the precipitate well stirred and shaken with fresh alcohol, then again spun down. The washing was repeated once more, then the precipitate (which seemed to have thixotropic properties) was transferred to a dish and dried in a

thin layer at 37° C. In one experiment, 40 litres of crude broth yielded in this way 23 g. of dirty white non-hygroscopic chalky solid.

The solid was stirred and shaken with water, of which the pH was adjusted to 8.0–8.5, and then centrifuged. After washing twice more with water at the same pH, the solid was inactive and was discarded. The strongest watery extract was treated with a small volume of phenol—perhaps 1 per cent.—and thoroughly shaken. An opalescence occurred, but if this did not develop into an easily centrifugable precipitate within half an hour or so, more phenol was added and the shaking was repeated. When a definite precipitate formed, the fluid was centrifuged, the supernatant was poured off and the active residue drained as fully as possible. From the supernatant, more active precipitate could be obtained by adding more phenol, but these subsequent crops were brown and semi-fluid, whereas the first was flocculent and pale cream in colour. The final supernatant contained much inactive material and was discarded. The various less active watery extracts and fractions were incorporated at suitable stages in the next batch to be worked up.

The first phenol precipitate was stirred up with water at pH 8.0, and shaken with an excess of ether, which removed the phenol. The watery solution sometimes threw down a precipitate on standing, which contained some activity, though most remained in the clear faint brown supernatant. This could be dried in a desiccator to a brittle glistening transparent brown non-hygroscopic glaze. Yield, 10–20 mg. per litre of crude broth. It was this material which was used for most of the animal experiments and bacteriological work.

PROPERTIES.

General properties.

The material is freely soluble in aqueous solutions at any pH, but is insoluble in all the usual organic solvents except acetic acid, and, to a small extent, pyridine; it is slightly soluble in moist phenol, for it can be eluted from charcoal with this reagent, although it is also precipitated by it from strong aqueous solution. It is odourless and has only a faint taste reminiscent of gelatine. On heating gradually on a slide it bubbles slightly, turns brown and then black, and gives a smell typical of burning protein. It is freely dialysable through cellophane. Solutions have a strong tendency to foam, and even with almost continuous addition of caprylic alcohol it was not possible to concentrate the material by distillation *in vacuo*. Foam separation was attempted, with a slow stream of compressed air as generator of foam, but although there was no destruction of colicine, there was no apparent concentration of it, either in the foam or mother liquor, when tests were carried out at pH 4.6, 5.0, 5.2 or 8.3. The active substance appears to be of a peptide nature; it is hoped to report on this at a future date.

Stability.

The activity is not appreciably reduced by boiling for half an hour at pH 2.0 or 7.0, but about 75 per cent of the activity is destroyed in half an hour at 100° C. and pH 9.0. No detectable activity remains after boiling for 25 minutes with $N/2$ hydrochloric acid or $N/10$ sodium hydroxide. The aqueous solution appears to keep its activity unimpaired in the ice chest for several months.

Destruction by enzymes.

Colicine is destroyed almost immediately by commercial pepsin or commercial trypsin. It is destroyed fairly rapidly by slices of mouse kidney, and more slowly by mouse liver. The supernatant from a centrifuged sample of staphylococcal pus slowly destroyed the activity of the substance when incubated with it.

Antibacterial properties.

Effect of inoculum size.—The diameter of the zone of inhibition in the cylinder-plate assay varied little with differences in the density of seeding of the plates, over a thousandfold range. A good dried preparation gave a zone of inhibition of *about* 18 mm. diameter at a dilution of 1 in 40,000. In broth culture, however, the titre was markedly affected by the size of inoculum, as shown in Table II.

TABLE II.—*Relation of Inoculum Size to End-point.*

Two-fold dilutions in Lemco broth. Test organism: sensitive *Bact. coli*.

Same sample of colicine used in all experiments.

Experiment No.	Dilution just preventing growth; 1 part in . . . million.		Inoculum size. Viable organisms per ml.
	After 24 hours.	After 48 hours.	
1	32	32	750
2	12·8	12·8	660
3	8	4	2,800
4a	6·4	1·6	4,000
b	6·4	6·4	"
c	3·2	3·2	"
d	6·4	3·2	"
5	6·4	6·4	approx. 2,000
6	3·2	3·2	15,000
7	2	1	approx. 400,000
8	0·05	0·05	15,000,000

Effect of serum.

The addition of up to 50 per cent serum with or without preliminary incubation made no noticeable difference to cylinder-plate assay values, but in dilution tests the presence of rather small amounts of serum definitely enhanced the potency of the substance, as shown in Table III. This effect did not appear to be related

TABLE III.—*Effect of Serum on End-point in Lemco Broth against Sensitive Bact. coli.*

Twofold dilutions.

Experiment No.	Inoculum size. Viable cells/ml.	Highest dilution just preventing growth. 1 in . . . million.		Final concentration of serum.
		In plain Lemco.	In Lemco + serum.	
1	—	6·4	25	10%
2	750	32	64	20%
3a	2,800	4	128	20%
3b	280,000	0·5	16	20%
4	—	2	8	10%

to pH, for the same end-point was obtained in plain Lemco broth whether the latter was buffered to pH 7.0 or to pH 7.4 (Abraham and Duthie, 1946). Serum heated to 56° C. for 30 minutes was as effective as raw serum.

Range of bacteria attacked.

In view of the marked effect of the inoculum size on the titre in broth, dilution tests on a variety of different organisms were not carried out. Instead, some idea of the relative sensitivity of different species was obtained from semi-quantitative gutter-plate experiments. Gutter plates were made containing graded concentrations of colicine in the gutter, and the various organisms being investigated were streaked across the plate perpendicular to, and on the surface of, the gutter itself. Such an experiment showed that a preparation which would inhibit the test *Bact. coli* for a distance of 8–10 mm. from the edge of the gutter could be diluted between 1600 and 3200 times before the same test organism would actually grow across the gutter. The following organisms were found to be at least 1000 times more resistant (possibly much more so) than the test *Bact. coli*;

Bact. coli N.C.T.C. No. 86; *Salm. typhi* (2 strains); *Bact. coli* strain CF1; *Sh. flexneri*; *C. xerosis*; *Myco. phlei*; El Tor vibrio; *Ps. pyocyanea*; *Salm. typhimurium*; *Salm. newport*.

Sh. shigae and *Sh. sonnei* were approximately as sensitive as the test *Bact. coli*.

Salm. enteritidis was about 60 times and *Salm. paratyphi A* about 100 times more resistant.

Mode of action on bacteria.—Sensitive bacteria are killed, not merely inhibited, but lysis has not been observed, even after prolonged incubation of sensitive and insensitive strains of bacteria in a solution containing 1 per cent of colicine. Incubation of cells from a young culture of sensitive *Bact. coli* in a 1 in 6000 solution of the substance in broth was not attended by any change in turbidity, and the nature of the zones of inhibition in the cylinder-plate test is what might be expected from a slowly diffusible non-lytic substance.

Sensitivity of pathogenic Bact. coli.—Nineteen strains of *Bact. coli* isolated from cases of bladder infection were tested for sensitivity to colicine by the streaking test. Seven were definitely inhibited, with nine there was a trace of inhibition, and with three none at all.

Action on red blood corpuscles.—Incubation of human red cells with a 1 in 100 solution of colicine in normal saline for 20 hours was accompanied by no more haemolysis than in saline alone.

Action of bacteria on colicine.—A solution of partially purified colicine was incubated at 37° C. for several hours with thick suspensions of living *Bact. coli*. The suspensions, which were heat-sterilised before re-assay, showed no diminution of activity with any of the strains tested, whether they were susceptible to the action of colicine or not.

Pharmacological properties.

Toxicity to mice.—Mice weighing 19 or 20 g. were injected intravenously with colicine dissolved in saline. Usually doses of 4 or 5 mg. (5 mice) had no effect; 8 or 10 mg. (3 mice) produced slight sickness with recovery after a few hours, and 18 mg. (1 mouse) also produced slight sickness. There was an exception,

however, with one batch of material, of which doses of 5, 10 and 20 mg. respectively to 3 mice produced increasing sickness and death in 8 hours or more, though 2.5 mg. had no effect.

*Toxicity to leucocytes in vitro.**—Human leucocytes were suspended in a solution containing colicine by the method previously described (Abraham *et al.*, 1941), and were watched for some hours. In 1 in 1000, the strongest concentration tested, the cells were as active throughout as in the control preparations.

Effect on smooth muscle.—Colicine was added to the Ringer-Locke solution in which was suspended the rhythmically contracting isolated horn of a guinea-pig uterus. No substantial effect on the contractions was produced by a concentration of 1 in 5000, the highest concentration tested.

Excretion.—Five mg. was injected intravenously into each of 2 mice, weighing respectively 23 and 25 g. The mice were killed 5 hours later and the urine tested on cylinder-plates. There was no activity. In another similar experiment, when 18.5 mg. were given, the urine gave a small but definite zone of inhibition, but the amount excreted was less than 5 per cent of the dose given. The material seems to have been largely destroyed in the body, which observation agrees with the results of the tissue slice experiments.

SUMMARY AND DISCUSSION.

That a variety of different bacterial inhibitors are formed by different strains of *Bact. coli* is evident from the differences in their reported heat stability, diffusibility, nature of action (lytic, bacteriostatic or bactericidal) and by the range of bacteria attacked. It is probable that in some cases the active agent is hydrogen peroxide. The strain forming the subject of this paper, strain CF1, an atypical *Bact. coli* Type I isolated from cat faeces, forms hydrogen peroxide under some conditions, but also a more specific inhibitor, possibly of a peptide nature, which appears to be different from those already reported as being formed by various strains of *Bact. coli*. The substance (called colicine) was formed under conditions of good aeration in Lemco or heart extract broth, but not in any of the synthetic media which were tried. At first it could only be demonstrated on a solid medium (Eijkmann, 1904; Foster and Woodruff, 1946), but later a good yield was obtained in broth if the culture was vigorously aerated. Foster and Woodruff had a similar experience with bacillin, formed by a *B. subtilis*, but in their case it was the addition of manganese to the medium, not aeration, which made production in fluid medium possible.

Colicine is not lytic, but it will kill a small inoculum of a sensitive organism at a dilution of 1 in several millions, though the inoculum size has a very considerable effect on the titre. In its action it is extremely specific, some strains of *Bact. coli* being among the most sensitive, other strains being unaffected. In this respect it resembles the specific lytic substance of Zamenhof (1945), but is sharply distinguished from it by its heat-stability and bactericidal action. (A preliminary investigation suggests that there may be many other inhibitors which are equally specific, formed by other strains of *Bact. coli*. These facts reinforce the feeling that must have been shared by other workers, that in the detection of antibiosis, much will depend on the choice of the test organisms—a choice hitherto guided largely by tradition or chance, and for which there is as

* Experiment done by Dr. M. A. Jennings.

yet no rational basis. Clearly an antibiotic as specific as colicine could easily escape detection if only a few test organisms were used.)

The potency of colicine is enhanced by serum, an effect which is believed to have been observed so far only in the case of gramicidin (Dubos, in press) and of an inhibitor formed by *B. subtilis* (Abraham and Gilliver, personal communication). Almost nothing is yet known of its chemical nature, except that it is freely soluble in water and behaves like a peptide, and that it is precipitated from concentrated crude aqueous solution by phenol. Impure as the dried product which has been obtained undoubtedly is, it has only a moderate toxicity to mice, and a remarkably low toxicity to leucocytes. It is non-haemolytic.

The substance is unlikely to be of any chemotherapeutic value as it is fairly rapidly destroyed in the body, and is also destroyed by at least some kinds of pus. It is most improbable that *Bact. coli* strain CF1 through its elaboration of colicine can exert any effect on the intestinal flora, as was postulated for some strains of *Bact. coli* by Nissle (1916), both because a liberal supply of oxygen appears to be necessary for the formation of the antibiotic, and also because the latter is destroyed almost immediately by trypsin and pepsin.

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